Analysis of Aflatoxin B_1 in Corn Using Capillary Electrophoresis with Laser-Induced Fluorescence Detection

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A capillary electrophoresis (CE) method was developed for the quantitation of aflatoxin B_1 (AFB_1) in corn. The instrumentation can be assembled easily from readily available components and takes advantage of the native fluorescence of AFB_1. Corn was extracted and aflatoxins were isolated using either silica column or affinity column cleanup procedures. Following cleanup, the analysis of each sample required 15 min: 10 min for the electrophoresis and 5 min for rinsing the capillary. The CE method was compared to an established HPLC method for the determination of AFB_1 in corn. The limit of detection by CE was 0.5 ppb, with a useful range of 1−100 ppb of AFB_1 in spiked corn. Recovery of AFB_1 averaged 85% over the range of 1−50 ppb (89% by HPLC). Forty naturally contaminated corn samples examined by using both methods showed good agreement ($r^2 = 0.969$). The reported CE method is suitable for the routine analysis of corn samples as an alternative to HPLC.

Keywords: *Mycotoxins; aflatoxin B_1; capillary electrophoresis; fluorescence; corn*

INTRODUCTION

The aflatoxins are a group of mycotoxins produced primarily by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*. They are found worldwide in a large variety of foods and commodities including corn, peanuts, and cottonseed. The potent carcinogenic potential of members of the aflatoxin family, in particular aflatoxin B_1 (AFB_1), has motivated extensive research into the occurrence of this toxin in food and the degree to which it represents a human health hazard. A wide variety of analytical methods have been developed for the aflatoxins since their discovery in the early 1960s. Methods have been developed using almost all of the current tools of analytical chemistry including high-performance liquid chromatography (HPLC), thin layer chromatography (TLC), gas chromatography/mass spectrometry (GC/MS), and capillary electrophoresis (CE). In addition, a variety of immunochemical methods such as enzyme-linked immunosorbent assays (ELISAs) have been developed for the aflatoxins and their metabolites that allow for the rapid screening for this group of toxins in corn [reviewed recently by Chu (1996)]. A number of automated systems using either reversed-phase solid phase extraction columns or affinity columns for isolation of the aflatoxins have been developed (Kussak et al., 1993; Jordan et al., 1994; Niedwetzki et al., 1994; Carman et al., 1996). An automated affinity liquid chromatography system has also been described (Urano et al., 1993).

Techniques for the isolation of AFB_1 from complex matrices as a preparatory step before application of the detection step have been described extensively. Several of the available cleanup methods were reviewed by Holcomb et al. (1992). In the present paper two commonly used techniques were used to isolate AFB_1 from contaminated corn: a silica gel column technique and an immunoaffinity column technique. The extracts were then analyzed by capillary electrophoresis with laser-induced fluorescence detection (CE-LIF). While there is a report of the use of CE for the analysis of aflatoxins (Cole et al., 1992), the limit of detection of the method (1000 ppb) did not have the sensitivity required to quantitate AFB_1 at the level of concern in the United States, which is 20 ppb in food destined for human consumption. We describe the application of CE-LIF to the analysis of AFB_1 in contaminated corn at levels as low as 0.5 or 1 ppb, respectively, using the two isolation techniques described.

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MATERIALS AND METHODS

Materials. Standard aflatoxins were purchased from Sigma Chemical Co. (St. Louis, MO): AFB_1 (lot 123H4039), AFB_2 (lot 123H4040), AFG_1 (lot 7H4048), AFG_2 (lot 83H4090). Deoxycholic acid, sodium borate, and trifluoroacetic acid (TFA) were also purchased from Sigma. Electrophoresis buffers were prepared from deionized (16.8 MΩ) water prepared with a Nanopure II purifier (Sybron/Barnstead). To prevent obstruction of capillaries, the electrophoresis buffers were passed through a 0.2 μm filter (Zapcap-CR, Schleicher & Schuell, Keene, NH) before use. All solvents were of HPLC grade; all other chemicals were of ACS reagent grade or better and were purchased from major suppliers.

Sample Preparation. A sample of control, low-aflatoxin corn was obtained by testing several lots from commercial sources. Corn was ground to pass a number 20 sieve using a Stein Mill and blended for 30 min with a Hobart mixer. Corn samples (50 g) were extracted according to AOAC Official Methods (AOAC, 1990) for aflatoxins in peanuts and peanut products (Method 968.22 parts A−E, also known as the "contaminants bureau" or "CB" method). HPLC was accomplished using AOAC Method 986.16E (aflatoxins M_1 and M_2 in fluid milk, liquid chromatographic method). The control corn used for recovery studies contained aflatoxin B_1 below the limit of detection of the HPLC method (0.1 ppb).

For spiking of control corn a 1.0 μg of AFB_1/mL standard was prepared in acetonitrile/benzene (2:98, v/v). The volume of spiking solution required to obtain corn containing 0.5−50 ng of AFB_1/g was added directly to the ground corn sample in the extraction flask. The samples were briefly shaken, before the addition of the Celite, water, and chloroform for the extraction procedure. Samples of naturally contaminated corn

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from a variety of sources that were stored at the USDA National Center for Agricultural Utilization Research (Peoria, IL) were extracted in the same manner.

A limited number of artificially contaminated samples were also tested using a commercially available affinity column cleanup procedure (Vicam LP, Watertown, MA). Fifty-gram samples of corn were extracted according to the manufacturer's instructions with 70% methanol/water (v/v). The equivalent of 1 g of corn was loaded onto the affinity column. The eluate from the column, in methanol, was dried under nitrogen gas.

Capillary Electrophoresis. The apparatus was assembled from readily available components. The capillary electrophoresis unit was a Beckman P/ACE 5000 equipped with a LIF detector. The commercial unit was modified by replacing the emission filters with a 400 nm long-pass filter and a 400 nm band-pass filter (Oriel Corp., Stratford, CT). The long-pass filter excluded light below 400 nm, while the band-pass filter transmitted light between 353 and 462 nm. The combination permitted collection of light between 400 and 462 nm. For experiments using the affinity column cleanup procedure, the 400 nm band-pass filter was removed to enhance sensitivity. Excitation light was provided by a 19 mW helium/cadmium laser (Model 3056, Omnichrome, Chino, CA) with 325 nm output.

Dried extracts of samples were reconstituted with 1.0 mL of electrophoresis buffer consisting of 50 mM sodium deoxycholate, 6 mM sodium borate, and 10 mM dibasic sodium phosphate, pH 9.1. For samples prepared by affinity column cleanup, the dried extract was reconstituted with 0.8 mL of electrophoresis buffer. A range of aflatoxin stock solutions from 0.5 to 100 μg/mL were prepared in acetonitrile, and the stock solutions were diluted 1:100 with electrophoresis buffer, yielding a range of standards from 5.0 to 1000 ng of AFB_1/mL for analysis. Standard solutions were prepared fresh daily to minimize effects from the potential degradation of AFB_1. Before injection of the sample or standard, the capillary (75 μm i.d., 57 cm total length, 50 cm length to detector) was rinsed with electrophoresis buffer for 2.0 min at 0.5 psi. The sample was injected for 5.0 s at 0.5 psi, equivalent to 30 nL. A voltage of 20 kV was applied, resulting in a current of approximately 104 μA. After 10 min, the voltage was removed and the capillary was rinsed for 1.5 min with 0.1 N sodium hydroxide and for an additional 1.5 min with deionized water. Data were collected and analyzed using Beckman system gold software (Fullerton, CA).

Fluorescence Spectra. For some experiments a scanning fluorometer, operated independently of the CE, was used to determine the effects of the electrophoresis buffer upon the excitation and emission spectra of AFB_1. Standard solutions of AFB_1 were scanned in either methanol, acetonitrile, or electrophoresis buffer using a SPEX FluoroMax fluorometer and DM3000 software (SPEX Industries, Inc., Edison, NJ).

RESULTS AND DISCUSSION

The use of CE for the analysis of mycotoxins in foods has been limited by the lack of published methods as well as by the expense of the technology. CE methods for mycotoxin standard solutions have been reported for the aflatoxins, ochratoxins, fumonisins, moniliformin, and zearalenone (Cole et al., 1991, 1992; Böhs et al., 1995; Maragos, 1995; Miyahara et al., 1996). The majority of the published methods have relied upon UV−visible absorbance as the means of detection. Because of the poorer sensitivity of such detection relative to fluorescence detection, there are very few methods published in which CE has been applied to the analysis of mycotoxins in foods (Maragos et al., 1996). There is a report of the application of CE-LIF to the analysis of AFB_1 in contaminated corn (Cole et al., 1992). The latter report provided an excellent description of the factors affecting the separation of aflatoxin standards by micellar electrokinetic capillary chromatography but included data for only a single sample containing 16 000 ppb of AFB_1. The limit of detection of that method was estimated to be 1000 ppb. Because the regulatory limit for AFB_1 in corn products destined for human consumption in the United States is 20 ppb, a method capable of measuring at, or below, this level was desired. In addition, it was desirable to construct the instrumentation from readily available components requiring minimal technical expertise to assemble.

The fluorescence of the aflatoxins has been used for many years as the means for detection of these compounds after separation by TLC or HPLC. Induction of fluorescence using a helium/cadmium laser for excitation was first described by Diebold and Zare (1977) as a means for detecting AFB_1 after separation by HPLC. Most of the literature on the fluorescence of the aflatoxins has dealt with the fluorescent properties in solvents such as methanol or solvent mixtures such as benzene/acetonitrile or toluene/acetonitrile. Each of the aflatoxins has a different fluorescence yield, and the commonly used HPLC methods require that the aflatoxins be derivatized before detection. Typically, the derivatization with a halogen such as bromine or iodine (Davis and Diener, 1980; Tuinstra and Haasnoot, 1983) or the reaction with TFA to form the hemiacetal (Büchi et al., 1966; Takahashi et al., 1977) enhances the fluorescence of AFB_1 and AFG_1 and therefore the sensitivity of the derivatization methods. More recently the association of aflatoxins with cyclodextrins has also been found to enhance sensitivity (Francis et al., 1988; Cepeda et al., 1996). While derivatization increases the sensitivity of the methods, derivatization was forgone with the CE method to speed up the analysis.

Because the fluorescence of AFB_1 may be influenced by the surrounding matrix, the effect of the electrophoresis buffer composition upon the fluorescence of AFB_1 was investigated. The electrophoresis buffer consisted of the bile salt deoxycholic acid, which forms micelles in solution (Cole et al., 1992). Figure 1 depicts the excitation and emission scans for a 1 μg/mL solution of AFB_1 in either methanol or electrophoresis buffer. The wavelength for the emission maximum of AFB_1, 427 nm, was identical when measured in either buffer or methanol using 360 nm excitation. However, the electrophoresis buffer caused a roughly 2-fold increase in the fluorescence intensity (Figure 1A). A similar effect was observed with the excitation spectrum: in this case the emission at 427 nm was maximum when the excitation wavelength was 360 nm (in methanol) or 350−400 nm (in electrophoresis buffer). However, unlike the emission maximum (427 nm), the excitation maximum in buffer slowly shifted over time relative to that seen in methanol. The observed shoulder at 400 nm in Figure 1B slowly increases over time, indicating possible decomposition of AFB_1 in electrophoresis buffer. To minimize the potential effects upon quantitation, standards were prepared fresh daily and samples were prepared within several hours of injection.

Separation of Aflatoxins. The four aflatoxins B_1, B_2, G_1, and G_2, which are neutral molecules, were separated from one another by the degree to which they interacted with the micelles in solution, an example of micellar electrokinetic capillary chromatography (MECC). The separation of the four aflatoxins from each other was adequate and was accomplished within 10 min (Figure 2). The order of migration of the aflatoxins with MECC was the same as that which has been commonly reported with reversed-phase HPLC (Francis, 1988;

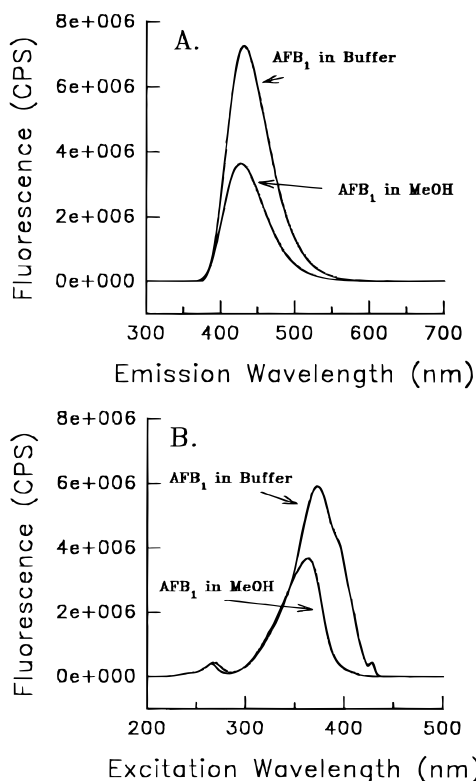

Figure 1. Influence of buffer composition on the fluorescence spectra of aflatoxin B_1: (A) emission spectra of 1 μg of AFB_1/mL in methanol or electrophoresis buffer (360 nm excitation); (B) excitation spectra of 1 μg of AFB_1/mL in methanol or electrophoresis buffer (427 nm emission).

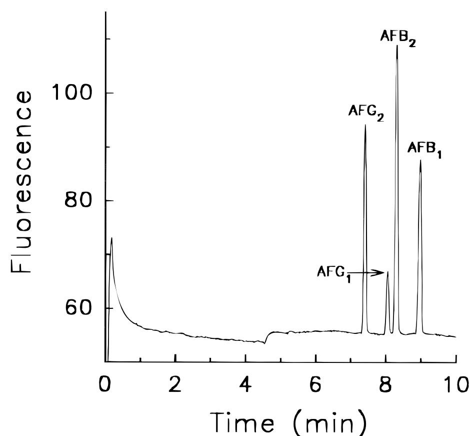

Figure 2. Separation of four aflatoxins by capillary electrophoresis. Aflatoxins were added to electrophoresis buffer at 75 ng/mL. Electrophoretic conditions are described in the text; 30 nL (2.25 pg of each toxin) was injected. The order of elution was AFG_2 (7.4 min), AFG_1 (8.0 min), AFB_2 (8.3 min), and AFB_1 (9.0 min).

Urano et al., 1993; Cepeda et al., 1996). For quantitation of AFB_1 in corn samples, AFB_1 standards were prepared in electrophoresis buffer over the concentration range of 5−1000 ng/mL. The response of the CE-LIF instrumentation to AFB_1 over this range is depicted in Figure 3 using MECC. The data in Figure 3 are the average from 12 separate standard curves. Each standard curve was prepared on a different day, and the variability shown is therefore likely to accurately reflect the repeatability of the standard curve between experiments.

Recovery of AFB_1 Added to Corn. Corn without detectable AFB_1 (<0.1 ppb by HPLC) was spiked with

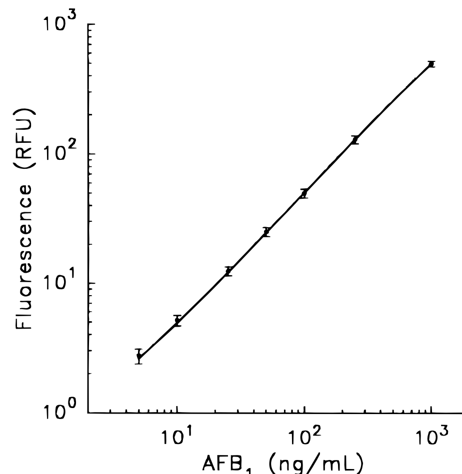

Figure 3. Fluorescence response of AFB_1 by CE-LIF. Standards were prepared by adding the appropriate amount of AFB_1 to electrophoresis buffer. Data represent the mean of 12 trials on 12 separate days. The error bars indicate ±2 SD from the mean. The curve is fit to a logistic dose−response equation ($r^2 = 0.999994$). The data also fit the line $y = a + bx$ with $a = 0.941$ and $b = 0.488$ ($r^2 = 0.999821$).

Table 1. Recovery of Added AFB_1 from Corn

AFB_1 added (ng/g)	% recovery by CE-LIF (± 1 SD[a])	% recovery by HPLC (± 1 SD)
0.5	NR[b]	67.3 ± 4.1[c]
1	90.3 ± 11.3	96.0 ± 1.7
2.5	67.3 ± 4.8	87.4 ± 5.0
5	86.3 ± 11.8	93.2 ± 5.7
10	84.7 ± 5.3	86.2 ± 3.2
20	84.1 ± 7.8	82.8 ± 8.7
50	98.7 ± 0.3	88.4 ± 5.1
overall	85.2	89

[a] SD, standard deviation. [b] NR, not reported; the concentration is below the limit of quantitation for the method. Data are the average of samples spiked in triplicate at each concentration level. [c] This value was excluded from the overall average to allow a direct comparison to the same concentration range (1−50 ppb) as the CE-LIF method.

AFB_1 over the range of 0.5−50 ppb. When the contaminants bureau (CB) method was used to isolate and concentrate AFB_1, the limit of detection for the method was 0.5 ppb (signal to noise ratio of 4). With the CB cleanup the equivalent of 10 g of corn was concentrated into 1.0 mL of electrophoresis buffer, 30 nL of which was injected. While it would be possible to further increase the sensitivity of the method by reconstituting the dried extract with less buffer (i.e. 0.1 mL instead of 1.0 mL), in practice the reproducibility of the sample injection suffered when sample vials containing <0.6 mL of solution were used for injection (data not shown). For a 0.5 ppb sample the concentration of AFB_1 in the solution injected into the CE was 5 ng/mL. To study the viability of the method, AFB_1 was added to corn over the range of 0.5−50 ppb. The spiked corn samples were extracted and analyzed by either CE-LIF or HPLC to determine recoveries. The recoveries using CE-LIF ranged from 67% to 98% (Table 1). The average recovery for 18 spiked samples was 85.2% by CE-LIF compared to 89% by HPLC. This indicates that with the same cleanup procedure the two methods yield similar results with spiked corn.

Because the CB method is laborious for the cleanup of multiple samples, the use of a more rapid affinity column method was also explored. The affinity columns used are commercially available (Vicam, LP) but have

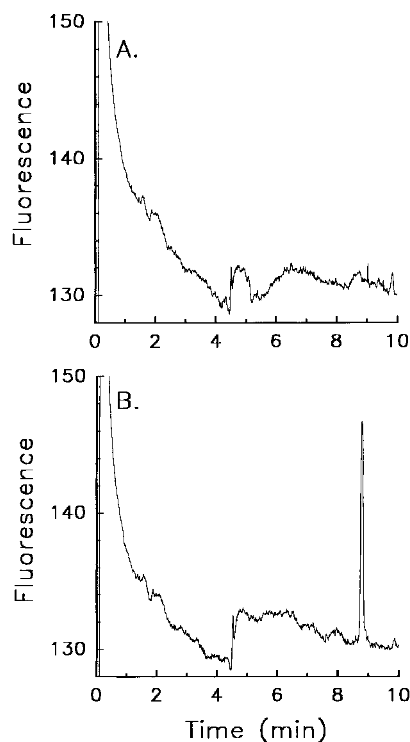

Figure 4. Electropherogram of a corn sample spiked with AFB_1 at 10 ppb and cleaned up using a commercially available affinity column: (A) control corn sample, containing <0.1 ppb of AFB_1; (B) corn spiked with 10 ppb of AFB_1.

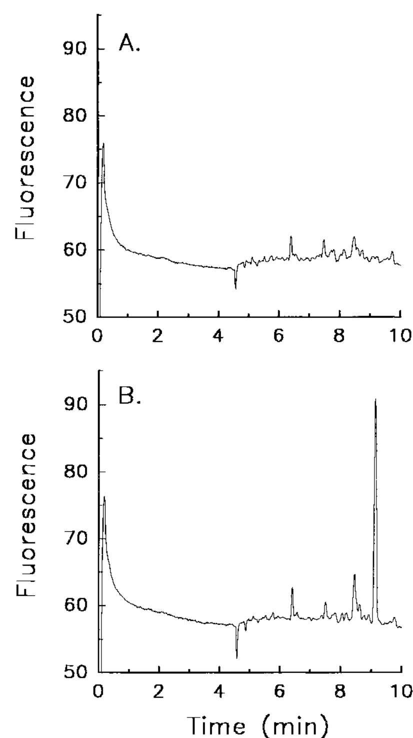

Figure 5. Electropherograms of corn samples after isolation of the AFB_1 with the CB method: (A) control corn containing <0.1 ppb of AFB_1; (B) corn naturally contaminated with 13.1 ppb of AFB_1.

the limitation in that less corn can be loaded onto the column than with the CB method (1 vs 10 g equivalent). This effectively reduced the sensitivity 10-fold when affinity columns were used. By eliminating the 400 nm band-pass filter, which attenuates the signal roughly 2-fold, and reducing the volume the extract was solubilized in before analysis (0.8 vs 1.0 mL), the method was able to be used for detecting moderate to low levels of AFB_1 contamination. The limit of detection with spiked corn using the affinity column cleanup was 1 ppb (signal to noise ratio of 4), with levels above 2.5 ppb giving an unambiguous peak (Figure 4). This sensitivity was only slightly worse than when the CB extraction was used (1 vs 0.5 ppb) in part because of the modifications to the instrumentation indicated earlier and in part due to the presence of fewer small peaks near AFB_1 that were observed with the CB cleanup.

Naturally Contaminated Samples. Having established that the CE-LIF method would detect AFB_1 isolated using either the CB method or affinity columns for cleanup, 40 naturally contaminated samples were assembled and tested. The samples were cleaned up using the CB method and then tested by CE-LIF or HPLC with fluorescence detection. The HPLC method involved derivatization with TFA, while the CE-LIF method did not. An electropherogram for a corn sample naturally contaminated with 13.1 ppb of AFB_1 is depicted in Figure 5. The comparison between the HPLC and CE methods, using the same cleanup, was good, with an $r^2 = 0.969$ for all 40 samples fit to the line $y = -0.16 + (1.135)x$ (Table 2). The slope of the line in Table 2 (1.135) indicated either a slight overestimation with the CE method or a slight underestimation by the HPLC method.

In conclusion, we have demonstrated the development of a CE-based system for the detection of AFB_1. The method can be used with either a silica gel column cleanup method (the CB method) or an immunoaffinity column cleanup method. Sensitivity is better with the silica gel cleanup (0.5 vs 1.0 ppb); however, the cleanup procedure is more tedious than using affinity columns. With either sample preparation, the method can be used to clearly quantitate AFB_1 at, or below, the regulatory limit of 20 ppb in corn. The method has similar cleanup requirements as available HPLC methods and the analytical (CE) step is similar in length to HPLC methods. Sensitivity with the CE-LIF method is slightly poorer than with current HPLC methods (0.5 vs 0.1 ppb). The advantage of the CE-LIF method over HPLC methods is the elimination of organic solvents for the determinative step. While this helps reduce solvent usage, additional extraction methods such as those that

Table 2. Comparison between HPLC and CE-LIF for the Determination of AFB_1 in Naturally Contaminated Corn Samples

sample	HPLC	CE-LIF	sample	HPLC	CE-LIF
1	ND[a]	ND	21	15.7	16.8
2	ND	ND	22	16.5	18.7
3	ND	ND	23	20.9	24.2
4	0.1	ND	24	22	24.1
5	0.2	0.7	25	26.7	36.0
6	0.2	ND	26	32.9	45.7
7	0.3	0.4	27	38.7	49.7
8	0.6	0.6	28	50.4	70.4
9	0.6	0.6	29	50.7	55.6
10	2.9	3.6	30	52.3	66.4
11	3.1	3.5	31	57.5	37.4
12	3.4	4.6	32	57.8	68.3
13	4.0	3.5	33	72.6	72.6
14	6.7	5.4	34	72.8	48.2
15	7.5	10.0	35	73.6	81.1
16	7.8	9.8	36	79.9	80.3
17	8.3	9.0	37	80.6	104.3
18	11.0	13.1	38	102.5	128.0
19	14.4	27.0	39	148.6	178.0
20	15.1	10.6	40	203.3	232.0

[a] ND, not detected, with a limit of detection of either 0.1 ppb (HPLC) or 0.5 ppb (CE-LIF).

use supercritical fluids (Taylor et al., 1993) could further reduce solvent consumption. The introduction of new CE hardware, including systems that can sample from 96 well plates and systems that can run several capillaries simultaneously, may further increase the utility of a rapid, determinative CE-LIF method.

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